AD_____

Award Number: W81XWH-13-1-0189

TITLE: Antibodies Expressed by Intratumoral B Cells as the Basis for a Diagnostic Test for Lung Cancer

PRINCIPAL INVESTIGATOR: Edward F. Patz, Jr., M.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27708-4677

REPORT DATE: June 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2015	Final	1Jul2013 - 31Mar2015

4. TITLE AND SUBTITLE
Antibodies Expressed by Intratumoral B Cells as the Basis for a Diagnostic Test for Lung Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0189

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Edward F. Patz, Jr., M.D.

E-Mail: patz0002@mc.duke.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Duke University
Durham, NC

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
As described in our Annual Report, our initial work focused on the expression, purification, and characterization of monoclonal antibodies (mAbs) cloned from intratumoral B lymphocytes (ITLs). While this work was successful in identifying a mAb with preferential binding to tropomyosin 4, all cloned mAbs exhibited polyreactivity. This suggested that the isolated ITLs that we used for mAb production were not members of clonal B cell populations arising in response to stimulation by intratumoral antigens. Hence, our most recent efforts have focused on methods to permit the cloning and expression of recombinant antibodies specifically from oligoclonal ITLs. We accomplished this by performing deep sequencing of VH and VL Ig genes from ITLs from seven additional lung cancer patients, We identified a total of 155,901 functional VH gene sequences with individual patient samples containing from 3,327 to 77,182 functional sequences. Although we obtained evidence for somatic hypermutation among these sequences, we did not identify any dominant clones. Sequences representing all VH gene families were identified and exhibited a distribution typically found in peripheral B cells. This suggests that it may be necessary to sequence Ig genes from microdissected intratumoral germinal centers in order to identify clonal ITL populations.

15. SUBJECT TERMS
intratumoral B lymphocytes, tumor antigens, lung cancer diagnosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

The aim of this research is to develop a blood test for autoantibodies that will determine whether a lung nodule discovered upon X-ray or CT is cancer. Our method is to first identify autoantibodies made by antigen stimulated, intratumoral B lymphocytes in patients with non-small cell lung cancer (NSCLC). These antibodies are then used to identify the stimulating antigens, and the antigens are used to formulate a diagnostic blood test for lung cancer.

Intratumoral B lymphocytes are associated with many types of cancer including NSCLC (1-3), breast cancer (4,5), and ovarian cancer (6). Analysis of B cells from tumors and metastatic lymph nodes found hypermutated antibody genes and expansion of class switched memory cells and plasmablasts in patients with bladder cancer (7). In addition, the presence of intratumoral germinal centers (GCs) have been reported in NSCLC (8,9), breast cancer (10-13), colon cancer (14), and germ cell cancers (15). In NSCLC, GCs are associated with early stage over late stage disease, suggesting a humoral immune response may hold these tumors in check (9).

Further characterization of antigen stimulated intratumoral B cells and identification of tumor antigens might be exploited for diagnostic or therapeutic purposes. As described in our Annual Report, we identified antibodies to tropomyosin 4 (TPM4) expressed by an intratumoral B cell of one lung cancer patient, confirmed the presence of the antibody in this patient's serum, and then performed a pilot study to determine if the serum autoantibody could be used as a diagnostic biomarker for lung cancer. Although promising, the TPM4 and other antibodies isolated at the same time were polyreactive, suggesting an absence of antigen stimulated clonal expansion in the ITLs we isolated. The current work employs deep sequencing in an attempt to identify clonal populations of ITLs for recombinant antibody production with the aim of increasing the likelihood of identifying antibodies against tumor antigens.

Body

Materials and Methods

Patients

The current study was approved by our Institutional Review Board and all patients signed an informed consent prior to enrollment in the study. Intratumoral lymphocytes (ITLs) used in this study were isolated from freshly resected NSCLC tissue from seven patients undergoing surgery at Duke University Medical Center. Patient demographics are shown in Table I.

B Cell Isolation and FACS

We isolated ITLs from lung tumor specimens by first placing the tissue in a Petri dish containing RPMI-1640 medium supplemented with 20 mM HEPES (RPMI/HEPES) and teasing the tissue into very small fragments with an 18 gauge needle. We then filtered the tissue fragment suspension sequentially through 100 μm and 40 μm pore-size nylon membranes and pelleted the cells by centrifugation for 10 min at 400 x g at 18°C. We resuspended the cell pellet in 2 ml RPMI/HEPES and isolated the lymphocytes over a Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) cushion according to the manufacturer's instructions. We resuspended the final lymphocyte pellet in Bambanker (Wako Chemicals, Richmond, VA) cell freezing medium and stored the cells at -80°C until FACS.

Prior to deep sequencing, thawed ITLs from each patient were first divided into approximately equal portions. One portion was used for DNA and RNA isolation and the other portion sorted as described below (Table 2).

For sorting of memory B cells and plasma cells, we washed the ITLs in PBS and stained them with Aqua vital dye (Invitrogen, Carlsbad, CA) and the following anti-human antibodies: CD3 phycoerythrin (PE)-Cy5, CD14 PE-Cy5, CD16 PE-Cy5, CD235a PE-Cy5, CD45 PE-Texas Red, CD19 allophycocyanin (APC)-Cy7, CD27 PE-Cy7, CD38 APC-Cy5.5, immunoglobulin M (IgM) FITC, and IgD PE (BD Biosciences, Mountain View, CA; Beckman Coulter, and Invitrogen). During the sort, we used forward- versus side-scatter gating to select for lymphocytes, and geometric gates to eliminate doublet events. We gated B cells as $CD45^{+}$, $CD3^{-}$, $CD14^{-}$, $CD16^{-}$, $CD235a^{-}$, and $CD19^{+}$; total memory B cells were further identified as IgD negative (IgD^{-}). B cells were sorted individually into 96-well PCR plates containing ice-cold PBS, DTT, and RNAsin (Promega, Madison, WI) and stored at -80°C until further processing. We performed FACS on a BD FACSAria (BD Biosciences, San Jose, CA) and analyzed the data with FlowJo (Tree Star, Ashland, OR).

Deep Sequencing

Results

Sorting of B cells from ITLs

In order to use deep sequencing as a means to identify clonally expanded B cells, with the aim of using this information to select specific B cells for recombinant antibody production, there had to be a way to access individual cells after the sequencing analysis. We accomplished this by dividing the ITL samples into two approximately equal aliquots, using one aliquot for sequencing and the other for fluorescence activated cell sorting (FACS) of individual B cells. Once sequences demonstrating oligoclonality were identified by sequencing B cells en masse, we could then go back to the sorted cells and sequence the VH and VL Ig genes from the individual cells. We would then prepare recombinant antibodies only from those cells whose VH and VL sequences placed them in the same clone(s) as those identified in the bulk sequencing analyses.

Details of the ITL samples used for sorting and DNA/RNA preps are shown in Table 2.

Deep Sequencing

We isolated and sorted ITLs from seven additional lung cancer patients as described above and subjected them to deep sequencing of Ig VH and VL genes.

Discussion

Tumor growth reflects a dynamic, multidimensional relationship between cancer cells and host cells, including stromal, endothelial, and immune cells (20,21). Tumors can be infiltrated with many types of lymphocytes, some that may foster and others that may inhibit tumor growth and progression (22). GCs, normally found in secondary lymphoid organs such as the spleen and lymph nodes, can also develop in tumors. GCs are well-defined loci of B cells, T cells and dendritic cells in which B cells proliferate and undergo differentiation, somatic hypermutation and affinity maturation (23). The presence of GCs in tumors is consistent with an *in situ* immune response to tumor antigens. We aimed to develop a method to identify the targets of antibodies produced by intratumoral B cells. This would allow us to explore the possibility that tumor antigens targeted by these intratumoral B cells may be relevant therapeutic targets or that the antibodies they produce may be diagnostic biomarkers. (See our Annual Report for additional information.)

In our Annual Report, we described how we used methodology originally developed to study the role of antigen in the progression of chronic lymphocytic leukemia (27) and later modified by researchers in the HIV

field to permit the cloning of human antibodies from antigen-secreting B cells (17,28). This study represented the first attempt to adapt this methodology to characterize single B cells isolated from a solid tumor and to identify their stimulating antigens. Although our results were promising and instructive, all of the recombinant antibodies that we produced from ITLs were polyreactive. Polyreactive antibodies are antibodies of low affinity (Kd of 10^{-3}-10^{-7} M) that react with a variety of totally unrelated antigens (18).

In newborns, 50% of cord B cells express polyreactive antibodies (32) and in adults, 15-20% of peripheral B cells express polyreactive antibodies (18). Interestingly, polyreactive antibodies in the circulation appear to have broad antimicrobial properties (33). It is intriguing to speculate that polyreactive antibodies in tumors may, in the aggregate, have antitumor properties. It is also possible, however, that the ITLs used for antibody production were present in the intratumoral space due to the presence of peripheral blood in the tumor vessels. In an attempt to improve our chances of isolating ITLs arising from antigen stimulated clonal expansion, our most recent work employed deep sequencing to identify those ITLs that have undergone clonal expansion and affinity maturation. We reasoned that since the VH and VL Ig gene sequences of polyreactive antibodies resemble germline antibodies, deep sequence analysis could distinguish monospecific from polyreactive antibodies expressed by intratumoral B cells.

This analysis identified a total of 155,901 functional VH gene sequences with individual patient samples containing from 3,327 to 77,182 functional sequences (Table 3). Sequences representing all VH gene families were identified and exhibited a distribution typically found in peripheral B cells. No dominant clones were identified.

Key Research Accomplishments

- Developed a method to quantify the distribution of VH gene families in ITLs using deep sequencing.
- Although no dominant clones were identified, the highly detailed characterization of ITL immunoglobulins provided by this analysis shows that this method can be an effective means of elucidating the humoral response within tumors.
- Using microdissected intratumoral GCs as a source of ITLs for VH and VL Ig gene sequencing may be a more effective starting point for identifying tumor associated antibodies for diagnostics and tumor associated antigens for therapeutics.

Reportable Outcomes

Manuscript submitted for publication (under review) - ***Interrogation of Intratumoral B Lymphocytes for the Discovery of Novel Molecular Targets***

Conclusions

The intratumoral space can be a rich source of information relating to the host immunological response to malignancy. As described in our Annual Report, we produced recombinant human antibodies from several ITLs isolated from freshly resected NSCLC tumors. Although all of these antibodies were polyreactive, one antibody demonstrated a preference for TPM4 and showed some selectivity as a diagnostic marker for lung cancer. This study provided the impetus for developing a method that would allow a more focused approach to identify intratumoral B cells responding to stimulation by tumor-associated antigens. Although we were successful in developing a robust deep sequencing protocol to characterize ITL VH and VL gene sequences,

we were unable to identify oligoclonal B cells. Hence, the next iteration of our protocol will be to focus on the deep sequencing of ITLs residing in intratumoral germinal centers (GCs). As we have reported (ref), GCs arise in tumors due to antigen stimulation and are more prevalent in early stage tumors. This suggests that the stimulating antigens responsible for intratumoral GCs may be important in a successful host anti-tumor immune response. Identifying clonally expanded B cells from these GCs will allow us to produce the associated antibodies, and identify the antigens to which these antibodies bind. Further studies will determine the utility of the antigens as therapeutic targets, and whether the antibodies can be used in therapeutic strategies. Alternatively, the antibodies may be useful as diagnostic agents.

References

1. Al-Shibli, K. I., Donnem, T., Al-Saad, S., Persson, M., Bremnes, R. M., and Busund, L. T. (2008) Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res* **14**, 5220-5227
2. Riemann, D., Wenzel, K., Schulz, T., Hofmann, S., Neef, H., Lautenschlager, C., and Langner, J. (1997) Phenotypic analysis of T lymphocytes isolated from non-small-cell lung cancer. *Int Arch Allergy Immunol* **114**, 38-45
3. Pelletier, M. P., Edwardes, M. D., Michel, R. P., Halwani, F., and Morin, J. E. (2001) Prognostic markers in resectable non-small cell lung cancer: a multivariate analysis. *Can J Surg* **44**, 180-188
4. Mahmoud, S. M., Lee, A. H., Paish, E. C., Macmillan, R. D., Ellis, I. O., and Green, A. R. (2012) The prognostic significance of B lymphocytes in invasive carcinoma of the breast. *Breast Cancer Res Treat* **132**, 545-553
5. Schmidt, M., Bohm, D., von Torne, C., Steiner, E., Puhl, A., Pilch, H., Lehr, H. A., Hengstler, J. G., Kolbl, H., and Gehrmann, M. (2008) The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Res* **68**, 5405-5413
6. Milne, K., Kobel, M., Kalloger, S. E., Barnes, R. O., Gao, D., Gilks, C. B., Watson, P. H., and Nelson, B. H. (2009) Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. *PLoS One* **4**, e6412
7. Zirakzadeh, A. A., Marits, P., Sherif, A., and Winqvist, O. (2013) Multiplex B cell characterization in blood, lymph nodes, and tumors from patients with malignancies. *J Immunol* **190**, 5847-5855
8. Dieu-Nosjean, M. C., Antoine, M., Danel, C., Heudes, D., Wislez, M., Poulot, V., Rabbe, N., Laurans, L., Tartour, E., de Chaisemartin, L., Lebecque, S., Fridman, W. H., and Cadranel, J. (2008) Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. *J Clin Oncol* **26**, 4410-4417
9. Gottlin, E. B., Bentley, R. C., Campa, M. J., Pisetsky, D. S., Herndon, J. E., 2nd, and Patz, E. F., Jr. (2011) The Association of Intratumoral Germinal Centers with Early-Stage Non-small Cell Lung Cancer. *J Thorac Oncol* **6**, 1687-1690
10. Coronella, J. A., Spier, C., Welch, M., Trevor, K. T., Stopeck, A. T., Villar, H., and Hersh, E. M. (2002) Antigen-driven oligoclonal expansion of tumor-infiltrating B cells in infiltrating ductal carcinoma of the breast. *J Immunol* **169**, 1829-1836
11. Kotlan, B., Simsa, P., Foldi, J., Fridman, W. H., Glassy, M., McKnight, M., and Teillaud, J. L. (2003) Immunoglobulin repertoire of B lymphocytes infiltrating breast medullary carcinoma. *Hum Antibodies* **12**, 113-121
12. Kotlan, B., Simsa, P., Teillaud, J. L., Fridman, W. H., Toth, J., McKnight, M., and Glassy, M. C. (2005) Novel ganglioside antigen identified by B cells in human medullary breast carcinomas: the proof of principle concerning the tumor-infiltrating B lymphocytes. *J Immunol* **175**, 2278-2285
13. Nzula, S., Going, J. J., and Stott, D. I. (2003) Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas. *Cancer Res* **63**, 3275-3280
14. Pages, F., Galon, J., Dieu-Nosjean, M. C., Tartour, E., Sautes-Fridman, C., and Fridman, W. H. (2010) Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* **29**, 1093-1102
15. Willis, S. N., Mallozzi, S. S., Rodig, S. J., Cronk, K. M., McArdel, S. L., Caron, T., Pinkus, G. S., Lovato, L., Shampain, K. L., Anderson, D. E., Anderson, R. C., Bruce, J. N., and O'Connor, K. C. (2009) The microenvironment of germ cell tumors harbors a prominent antigen-driven humoral response. *J Immunol* **182**, 3310-3317
16. Tiller, T., Meffre, E., Yurasov, S., Tsuiji, M., Nussenzweig, M. C., and Wardemann, H. (2008) Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* **329**, 112-124
17. Liao, H. X., Levesque, M. C., Nagel, A., Dixon, A., Zhang, R., Walter, E., Parks, R., Whitesides, J., Marshall, D. J., Hwang, K. K., Yang, Y., Chen, X., Gao, F., Munshaw, S., Kepler, T. B., Denny, T., Moody, M. A., and Haynes, B. F. (2009) High-throughput isolation of immunoglobulin genes from single human B cells and expression as monoclonal antibodies. *Journal of virological methods* **158**, 171-179
18. Notkins, A. L. (2004) Polyreactivity of antibody molecules. *Trends Immunol* **25**, 174-179
19. Mano, H. (2012) ALKoma: a cancer subtype with a shared target. *Cancer Discov* **2**, 495-502

20. Christensen, J. D., Colby, T. V., and Patz, E. F., Jr. (2010) Correlation of [18F]-2-fluoro-deoxy-D-glucose positron emission tomography standard uptake values with the cellular composition of stage I nonsmall cell lung cancer. *Cancer* **116**, 4095-4102
21. Mantovani, A., Garlanda, C., and Allavena, P. (2010) Molecular pathways and targets in cancer-related inflammation. *Ann Med* **42**, 161-170
22. DeNardo, D. G., Andreu, P., and Coussens, L. M. (2010) Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev* **29**, 309-316
23. MacLennan, I. C. (1994) Germinal centers. *Annu Rev Immunol* **12**, 117-139
24. Chen, Y. (2004) Identification of human tumor antigens by serological expression cloning: an online review on SEREX *Cancer Immunity*
25. Punt, C. J., Barbuto, J. A., Zhang, H., Grimes, W. J., Hatch, K. D., and Hersh, E. M. (1994) Anti-tumor antibody produced by human tumor-infiltrating and peripheral blood B lymphocytes. *Cancer Immunol Immunother* **38**, 225-232
26. Yasuda, M., Takenoyama, M., Obata, Y., Sugaya, M., So, T., Hanagiri, T., Sugio, K., and Yasumoto, K. (2002) Tumor-infiltrating B lymphocytes as a potential source of identifying tumor antigen in human lung cancer. *Cancer Res* **62**, 1751-1756
27. Volkheimer, A. D., Weinberg, J. B., Beasley, B. E., Whitesides, J. F., Gockerman, J. P., Moore, J. O., Kelsoe, G., Goodman, B. K., and Levesque, M. C. (2007) Progressive immunoglobulin gene mutations in chronic lymphocytic leukemia: evidence for antigen-driven intraclonal diversification. *Blood* **109**, 1559-1567
28. Morris, L., Chen, X., Alam, M., Tomaras, G., Zhang, R., Marshall, D. J., Chen, B., Parks, R., Foulger, A., Jaeger, F., Donathan, M., Bilska, M., Gray, E. S., Abdool Karim, S. S., Kepler, T. B., Whitesides, J., Montefiori, D., Moody, M. A., Liao, H. X., and Haynes, B. F. (2011) Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. *PLoS One* **6**, e23532
29. Birring, S. S., and Peake, M. D. (2005) Symptoms and the early diagnosis of lung cancer. *Thorax* **60**, 268-269
30. Moody, M. A., Yates, N. L., Amos, J. D., Drinker, M. S., Eudailey, J. A., Gurley, T. C., Marshall, D. J., Whitesides, J. F., Chen, X., Foulger, A., Yu, J. S., Zhang, R., Meyerhoff, R. R., Parks, R., Scull, J. C., Wang, L., Vandergrift, N. A., Pickeral, J., Pollara, J., Kelsoe, G., Alam, S. M., Ferrari, G., Montefiori, D. C., Voss, G., Liao, H. X., Tomaras, G. D., and Haynes, B. F. (2012) HIV-1 gp120 vaccine induces affinity maturation in both new and persistent antibody clonal lineages. *J Virol* **86**, 7496-7507
31. Moody, M. A., Zhang, R., Walter, E. B., Woods, C. W., Ginsburg, G. S., McClain, M. T., Denny, T. N., Chen, X., Munshaw, S., Marshall, D. J., Whitesides, J. F., Drinker, M. S., Amos, J. D., Gurley, T. C., Eudailey, J. A., Foulger, A., DeRosa, K. R., Parks, R., Meyerhoff, R. R., Yu, J. S., Kozink, D. M., Barefoot, B. E., Ramsburg, E. A., Khurana, S., Golding, H., Vandergrift, N. A., Alam, S. M., Tomaras, G. D., Kepler, T. B., Kelsoe, G., Liao, H. X., and Haynes, B. F. (2011) H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. *PLoS One* **6**, e25797
32. Chen, Z. J., Wheeler, C. J., Shi, W., Wu, A. J., Yarboro, C. H., Gallagher, M., and Notkins, A. L. (1998) Polyreactive antigen-binding B cells are the predominant cell type in the newborn B cell repertoire. *Eur J Immunol* **28**, 989-994
33. Zhou, Z. H., Zhang, Y., Hu, Y. F., Wahl, L. M., Cisar, J. O., and Notkins, A. L. (2007) The broad antibacterial activity of the natural antibody repertoire is due to polyreactive antibodies. *Cell Host Microbe* **1**, 51-61
34. Lleo, A., Invernizzi, P., Gao, B., Podda, M., and Gershwin, M. E. (2010) Definition of human autoimmunity--autoantibodies versus autoimmune disease. *Autoimmun Rev* **9**, A259-266

Supporting Data

Characteristic	Value
Age (yr)	
Mean ± SD	71.7 ± 12.5
Gender:	
Female	3
Male	4
Tobacco use:	
Pack-years	32.6 ± 18.3
Tumor size (cm)	
Mean ± SD	4.5 ± 1.6
Pathologic stage:	
IA	1
IB	3
IIA	0
IIB	1
IIIA	1
IIIB	0
IV	1
Cell type:	
Adeno	4
Squamous cell	2
LCLC	1

Table 1. Patient Demographics for deep sequencing of VH and VL Ig genes in NSCLC ITLs.

Sample ID	# cells recovered	% viability	# cells sorted	Frequency of B cells from Total (%)	# cells used for DNA/RNA isolation	Isolated DNA (ng/µL)	Isolated RNA (ng/µL)
11-019T	1.09E+06	93.8	5.00E+05	0.13	5.40E+05	7.7	3.2
11-021T	2.47E+06	42	1.11E+06	3.6	1.26E+06	29.5	13
11-057T	1.40E+06	90.8	5.97E+05	0.09915	7.86E+05	11.6	21
11-060T	4.00E+05	51	1.56E+05	1.04	2.00E+05	10.5	1.9
12-001T	1.62E+06	81.5	8.54E+05	6.84	7.18E+05	16.7	2
12-002T	1.48E+07	80	5.95E+06	0.01715	7.81E+06	17.8	1.3
12-017T	4.55E+05	75	1.84E+05	5.51	2.20E+05	3.5	0.4

Table 2. Details of ITL sorting and DNA/RNA isolation for deep sequencing.

	Functional VH Gene Sequences							
Sample ID	VH1	VH2	VH3	VH4	VH5	VH6	VH7	TOTAL
11-019	7,822	38	4,921	3,621	3,015	8	0	19,425
11-021	14,871	665	26,292	21,490	13,681	181	2	77,182
11-057	1,029	93	1,436	691	64	14	0	3,327
11-060	9,469	35	9,213	798	75	4	190	19,784
12-001	5,428	225	10,142	3,427	875	93	0	20,190
12-002	8,640	64	3,117	2,302	844	265	761	15,993
12-017	5,466	220	4,995	2,556	1,686	65	0	14,988

Table 3. Functional VH gene sequences identified in each VH gene family after deep sequencing of ITL Ig genes from 7 patients.